IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 6691
Willem F. WOLKERS et al.	Examiner: Leon B. Lankford, Jr.
Application No.: 10/802,099	Technology Center/Art Unit: 1651
Filed: March 16, 2004	DECLARATION OF DR. FERN TABLIN
For: THERAPEUTIC PLATELETS AND METHODS	
Customer No.: 20350	

Commissioner for Patents
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I, DR. FERN TABLIN, hereby declare and state:

1. I received a B.A. degree in history and biology from the University of Pennsylvania in 1973. I received a Doctor of Veterinary Medicine degree, cum laude, from the University of Pennsylvania in 1980, and a Ph.D. from the University of Pennsylvania in 1984. From 1984 to 1985, I served as a Lecturer in the Department of Clinical Studies, School of Veterinary Medicine, the University of Pennsylvania. Since 1985, I have been a faculty member of the Department of Anatomy, School of Veterinary Medicine, University of California at Davis. I was an assistant professor from 1985 to 1992, and an associate professor from 1992 to 1999. Since 1999, I have been a full professor in the department. From 2003 to 2005, I was also co-director with Dr. John Crowe of the University of California's Center for Biostabilization, which was focused on the problems of stabilizing (a) platelets, (b) red blood cells and, (c) nucleated mammalian cells.

2. Since 1985, a major portion of my research has focused on membrane cell biology, and more particularly on methods for stabilizing animal and human platelets. In the past decade, I have been the principal investigator or an investigator on grants totaling millions of dollars for research on platelet stabilization. I am a co-author of dozens of publications in the peer-reviewed scientific literature in this area and have given invited talks at both national and international meetings on platelet stabilization, including loading platelets with trehalose. I have also served as a reviewer of a number of publications on aspects of membrane and cell biology, including Nature (Cell Biology), the Journal of Biological Chemistry, and Blood, and am a Fellow of the American Association for the Advancement of Science. By virtue of my education, experience, and research, I am knowledgeable about platelet stabilization, including methods that involve loading platelets with trehalose prior to drying them. A copy of my curriculum vitae is attached as Attachment 1.

3. To briefly review aspects of platelet biology relevant to the present discussion, platelets originate by budding off from megakaryocytes and, if not activated, last about 10 days in the circulation. As fragments of cells, platelets do not have a nucleus, although they do have some organelles, such as mitochondria. In particular, they contain secretion vesicles known as alpha granules, which contain thrombospondin and a number of growth factors. Platelets in the circulation are considered to be a "resting" (unactivated) state: they retain a discoid shape and the secretory vesicles remain in the cytoplasm. Certain circumstances, including contact with agents such as thromboxane A2, ADP, and thrombin, trigger platelets to activate. Activation causes a number of physiological changes in a platelet, including exocytosis of the alpha granules, reorganization of the platelet membrane, activation of a membrane enzyme, thromboxane A2, and a change in platelet shape from discoid into a more irregularly shape, which can include pseudopods. Activated platelets bind fibrinogen, which ultimately binds platelets together, and tend to recruit more platelets into what is known as a platelet plug. The surface of activated platelets becomes a site for the complex enzyme interactions underlying the coagulation cascade. The presence of activated platelets in the circulation is known to affect leukocyte trafficking.

4. Thus, activated platelets can be used where there is an immediate need for hemostasis. For example, dried activated platelets can be used on a bandage, where rehydration by fluids from a wound will reconstitute the platelets and place their coagulation-promoting factors in contact with the wound. On the other hand, activated platelets are less satisfactory for infusion into the circulation. They are prothrombotic and may cause clotting in inappropriate vessels. They are also removed from the circulation faster than are resting (unactivated) platelets and are thus less able to contribute to maintaining normal clotting activity over time. Thus, while resting platelets can be used anywhere activated platelets can be used (since they will activate if contacted with the usual triggers that initiate normal platelet activation), activated platelets cannot be used everywhere resting platelets can be used.

5. I am aware that Roser et al., U.S. Patent No. 6,221,575, has been cited against the present patent application. Roser teaches the following with respect to introducing trehalose into platelets:

> Suitable methods include, but are not limited to, electropermeabilisation, phase transition of the membrane, osmotic methods such as the use of organic osmolytes and pinocytosis, transient lysis methods such as acid shock and reversible cross-linking and the use of membrane permeable, esterase-labile trehalose derivatives. Effective means of electropermeabilisation are described for instance in the Examples herein and in Hughes and Crawford (1989), Biochemica Biophysica Acta 981:277-287; and Hughes and Crawford (1990) 634th Meeting, Bath, Biochemical Society Transactions 871-873. Effective means of phase transition of the membrane for platelet loading with trehalose are described for instance in Oliver et al. The Bethesda Meeting. Effective means of pinocytosis are described for instance in Okada and Rechsteiner (1982) Cell 29: 33; and Rechsteiner (1987) Methods in Enzymology 149: 42. Effective means of transient lysis are described for instance in Magnani et al. (1992) Proc. Natl. Acad Sci. 89:6477; Ihler and Tsang 1987 Methods in Enzymology 149: 221; and Dale (1987) Methods in Enzymology 149:229.

Roser, '575 patent, at column 5, lines 17-41.

6. The Roser '575 patent does not teach a method of getting trehalose into platelets in amounts sufficient to stabilize the platelets for freeze-drying and rehydration without activating the platelets. The methods that Roser teaches for loading platelets with trehalose are the following: electropermeabilisation, by transient lysis methods, by osmotic methods such as pinocytosis, and by phase transition. I will address each of these in turn.

7. Electropermeabilisation, the only method the Roser patent uses in its Examples, is known to activate platelets. It is therefore incapable as a method of providing a trehalose-loaded, resting platelet.

8. Transient lysis does not activate platelets, but neither is it satisfactory as a means for obtaining trehalose-loaded, resting platelets that are stabilized enough to maintain clotting functionality after being freeze-dried and rehydrated. Transient lysis has at least two problems. First, it makes holes in the membrane. These holes can reseal, but can also promote leakage of small molecules in and out of the cell. This leakage makes the use of transient lysis problematic. For example, if divalent cations are present in the medium, they may be able to leak in and activate the platelet. Second, it is very unlikely that the holes in the membrane exist long enough to permit enough trehalose to be loaded into the platelet to stabilize the platelet for procedures such as freeze-drying. Thus, I do not believe that transient lysis provides a method for providing resting platelets loaded with sufficient trehalose that they will, for example, respond to thrombin by clotting after being freeze-dried and rehydrated.

9. The Roser patent mentions the use of "osmotic methods such as the use of organic osmolytes and pinocytosis." The "pinocytosis" to which Roser refers, however, is not what is commonly thought of by biologists as "pinocytosis" but is rather a so-called "osmotic lysis" technique taught in two references by Rechsteiner, in which L929 cells are subjected to osmotic manipulations to introduce macromolecules into cellular cytoplasm.

10. L929 cells are nucleated, murine cells commercially available from Sigma-Aldrich (St. Louis, MO). In Rechsteiner's osmotic lysis procedure, the cells were first incubated in a hyperosmotic, 0.5 M sucrose solution, which shrank the volume of the cell cytoplasm, and then subjected to a hypotonic culture medium, which caused the volume of the cell cytoplasm to expand. Any pinosomes that entered the cytoplasm from the hypertonic solution then burst due to the osmotic difference, and released their contents. While pinocytosis occurred in the osmotic lysis technique taught in the Rechsteiner references, based on my years of experience as a reviewer and researcher, I believe a cell biologist would not call the osmotic lysis technique "pinocytosis" without also adding a qualifying descriptor to explain what the technique really involves. The two Rechsteiner references referred to in the Roser patent are provided as Attachments 2 and 3 to this Declaration.

11. Nucleated mammalian cells, such as the L929 cells used in the Rechsteiner papers, are much larger than platelets and have far more membrane surface. They are therefore much more able to withstand the osmotic manipulations necessary to practice the osmotic lysis method taught by Rechsteiner. Given my experience in working with platelets over almost two decades of research, I believe that subjecting platelets to the osmotic manipulations taught by Rechsteiner would activate them.

12. Roser also mentions that trehalose can be loaded into platelets by phase transition, and cites as support a presentation by Ann Oliver at a 1996 meeting in Maryland (the so-called "Bethesda Meeting," see, Roser, col. 2, lines 4-8). Ms. Oliver is one of the inventors named on the present application. The phase transition Ms. Oliver would have been talking about in 1996 was the phase transition that platelets undergo between 12° and 20° C, which was the only phase transition in platelets known at that time. See, specification, at page 6, lines 21-22. Unfortunately, platelets at this phase transition temperature do not take up enough trehalose to stabilize them during drying. Moreover, platelets that have been chilled through this phase transition have been shown to be damaged and to have reduced time in the circulation.

13. As also discussed in the specification, however, it was found that platelets have a second phase transition between 30° and 37° C. Surprisingly, platelets incubated with trehalose at the concentrations described in the specification and at or between these temperatures take up trehalose in quantities sufficient to permit them to be dried and rehydrated without activating them. See, specification, at e.g., page 6, lines 22-23, and Figure 1. Further, the platelets are able to clot when incubated with thrombin for three minutes at physiological temperature, 37° C. See, specification, at page 23, lines 19-24.

14. In short, Roser does not teach a method permitting resting platelets to be loaded with amounts of trehalose sufficient to permit them to provide clotting activity after being freeze dried and then rehydrated. The present invention provides a simple but effective solution for what has been a long known problem in the art.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

12/20/05

Date

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The ability to successfully deglycosylate any protein or enzyme in order to specifically target a selected cell type requires that there be (1) an available source of pure enzyme; (2) specific exoglycosidases of high specific activity available either commercially or relatively easily purified; (3) chemical or biochemical means available for the testing of the product, preferably at each step; and (4) a means of separating the glycosidases used from the desired enzyme product.

The characteristic and unique accumulation of glucocerebroside only in cells of the monocyte–histiocyte series, makes Gaucher's disease an excellent prototype for the study of enzyme replacement therapy. The principles demonstrated for the enzymatic deglycosylation of glucocerebrosidase may be applied to the cell-specific delivery of other glycoproteins as well. Other lysosomal diseases in which storage occurs in multiple cell types may be ameliorated by administration of macrophage-directed enzymes if, by so doing, storage material can be digested during the normal phagocytic turnover of senescent cells. Consideration of the kinetics of degradation and the structural features affecting the stability of enzymes *in vivo* are prerequisites to improving the bioengineering of targeted lysosomal enzymes. Animal and culture models have been developed for the study of glucocerebrosidase delivery to specific cell types and substrate degradation. Other studies have progressed toward a definition not only of the receptors at the plasma membrane involved in the internalization of exogenous enzyme, but also of internal receptors or properties of the lysosome responsible for intracellular protein trafficking. A complete understanding of the forces acting to direct endogenous or exogenously supplied enzyme to a given subcellular organelle will require a synthesis of experimental results from all areas of glycoprotein research.

Acknowledgment

I would like to thank Mr. Mark Garfield, Mr. Gregory Zirzow, and Dr. F. Scott Furbish for help in developing some of the basic methods, and Dr. John Barranger for reading and criticizing the manuscript.

[4] Osmotic Lysis of Pinosomes

By MARTIN RECHSTEINER

Introduction

Most cells exhibit substantial rates of fluid-phase pinocytosis, one aspect of the membrane traffic between the Golgi apparatus, endosomes,

and plasma membrane.[1] This shuttle of membrane vesicles provides the opportunity to introduce macromolecules into the cytoplasm by selectively breaking the vesicles after they form at the plasma membrane. In principle, any manipulation introducing holes into pinocytic vesicle membranes while sparing the plasma membrane would provide a method for transferring molecules from the extracellular fluid to the cytoplasm. Early attempts to lyse pinocytic vesicles using photoactive dyes proved unsuccessful, but eventually a method was developed in which osmotic pressure caused selective rupture of the pinocytic vesicle–endosome compartments.[2]

Briefly, the procedure is performed as follows. Cells are first incubated in culture medium containing high concentrations of sucrose, polyethylene glycol, and the molecule to be transferred. Even though the cells shrink considerably due to loss of water to the hypertonic medium, pinocytosis continues. The external sucrose does not pass through the plasma membrane, but it does fill those pinocytic vesicles formed during exposure to the hypertonic medium. After several minutes, the cells are transferred to culture medium that has been diluted with 0.66 parts water. The shrunken cytoplasm swells to more than its original volume, and when this occurs, the sucrose-laden pinocytic vesicles become surrounded by hypotonic cytosol. Consequently, they swell and break from the increased osmotic pressure, thereby releasing their contents to the cytosol (see Fig. 1 for a schematic diagram).

The efficiency of the procedure is related to the rate of pinocytosis and the volume occupied by the vesicle–endosome compartment. Some cells exhibit high rates of fluid-phase pinocytosis with the pinosome–endosome compartment occupying as much as 3% of the total cell volume.[3] Such cells are ideal for the use of pinosome lysis. However, all cells carry out pinocytosis to some degree, so the procedure should prove suitable for most cell types. Osmotic lysis of pinosomes has already been used with a number of cultured cell lines to study intracellular proteolysis,[4,5] cytoplasmic pH changes,[6,7] Ca^{2+} movements,[8] and uptake of toxins,[9] and to introduce antibodies into the cytoplasm for studies on microtubule function.[10]

[1] R. M. Steinman, I. S. Mellman, W. A. Muller, and Z. A. Cohn, *J. Cell Biol.* **96**, 1 (1983).
[2] C. Y. Okada and M. C. Rechsteiner, *Cell* **29**, 33 (1982).
[3] R. M. Steinman, S. E. Brodie, and Z. A. Cohn, *J. Cell Biol.* **68**, 665 (1976).
[4] D. T. Chin, L. Kuehl, and M. Rechsteiner, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5857 (1982).
[5] R. Hough and M. Rechsteiner, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 90 (1984).
[6] P. Rothenberg, L. Glaser, P. Schlesinger, and D. Cassel, *J. Biol. Chem.* **258**, 4883 (1983).
[7] D. Cassel, P. Rothenberg, Y.-X. Zhuang, T. F. Deuel, and L. Glaser, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6224 (1983).
[8] M. B. Hallett and A. K. Campbell, *Immunology* **50**, 487 (1983).
[9] P. C. Ghosh, R. B. Wellner, and H. C. Wu, *Mol. Cell. Biol.* **4**, 1320 (1984).
[10] S. I. Vogt, J. A. Snyder, and J. M. Mullins, *J. Cell Biol.* **97**, 191a (1983).

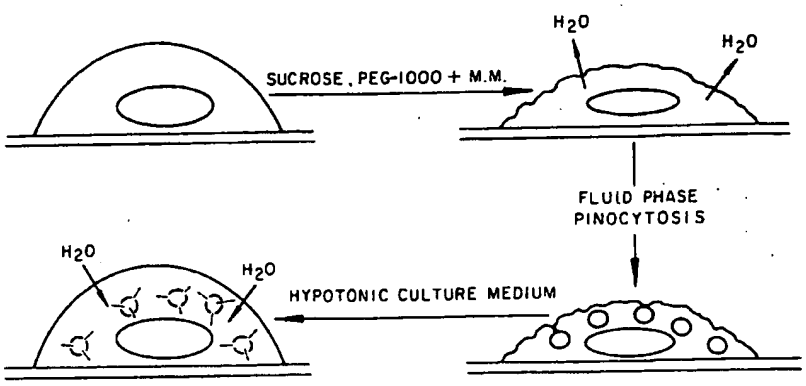

FIG. 1. Schematic representation of osmotic lysis of pinocytic vesicles.

Solutions Required

The necessary solutions are very easy to prepare. Hypertonic medium consists of tissue culture medium (there is no evidence that the specific formulation is crucial) supplemented to 0.5 *M* with sucrose, 10% with polyethylene glycol 1000 (PEG-1000). We normally use 10 m*M* HEPES as a buffer so that the medium pH is reasonably constant during the manipulations required for osmotic lysis. The concentration of sucrose may be increased to 1 *M* without serious harm to L929 cells, but 0.5 *M* is sufficient. Polyethylene glycol 1000 is required for two reasons. First, rates of pinocytosis are increased substantially in the presence of PEG-1000. Second, macromolecules do not escape pinocytic vesicles unless PEG-1000 is present. The small water-soluble fluorescent compound lucifer yellow can escape pinosomes after osmotic lysis in the absence of PEG-1000, so we speculate that PEG-1000 perturbs the membrane so that larger or more stable pores form during osmotic lysis in its presence. Fetal calf serum is also important because rates of pinocytosis are markedly reduced in serum-free medium.

The molecule to be transferred should be dissolved directly in hypertonic medium. Since the amount of any macromolecule internalized by fluid-phase pinocytosis is directly proportional to its concentration in the medium, the macromolecule should be dissolved in the smallest volume of medium possible to provide maximal transfer. It is possible to retrieve much of the hypertonic solution after use and, therefore, repurify the macromolecule from the hypertonic medium.

Hypotonic solution is prepared by diluting ordinary culture medium with distilled water. L929 cells will survive transient (< 5 min) exposure to

medium diluted with four parts water, but it is only necessary to dilute medium with 0.66 parts water to achieve substantial lysis of pinocytic vesicles.

Osmotic Lysis Procedure

All operations are carried out at 37° to maximize rates of pinocytosis in cultured mammalian cells. The procedure can be employed with cells in monolayer or suspension. In either case the transfer of solutions should be done quickly to achieve the maximum osmotic pressure gradient. Almost 0.5 ml of medium may remain on a well-drained tissue culture dish, and this is enough to cause failure if small volumes of hypertonic medium are used. Therefore, before we incubate cells in the final hypertonic solution containing the macromolecule to be transferred, we routinely rinse monolayers twice with 2 ml of hypertonic solution lacking the macromolecule. Because the goal is usually the transfer of as many molecules as possible to the cytoplasm, the final hypertonic rinse should be thoroughly removed before adding the hypertonic medium containing the macromolecule. We generally add 0.2 ml for 60-mm petri dishes and 1.0 ml for 150-mm dishes.

After incubating the cells for 10 min at 37° in hypertonic medium containing the molecule to be transferred, the hypertonic medium should be drained and removed with a pasteur pipette. As mentioned above, hypertonic medium containing the macromolecule may be reused in diluted form or the molecule can be repurified. At this time the plates should be rinsed quickly with two 3- to 5-ml portions of hypotonic medium and then allowed to sit in hypotonic medium for 2 min. The two rapid rinses with hypotonic medium remove the layer of hypertonic medium surrounding the cells. If the layer of viscous hypertonic medium is not first rinsed away, a sufficient osmotic pressure gradient is not formed, and the pinocytic vesicles will not break. Following this brief exposure to hypertonic medium the cells can be rinsed in normal culture medium and studied as desired.

Osmotic lysis of pinosomes can also be effected on cells in suspension provided they exhibit pinocytosis. Based on qualitative histochemical assays we have found that the osmotic lysis procedure works well on freshly trypsinized L929 cells.[2] The procedure is actually more efficient in suspension due to a lower volume ratio of medium to cells. Freshly trypsinized cells or cells that normally grow in suspension are collected by centrifugation in a 15-ml plastic cinical centrifuge tube. The overlying medium is removed and the cell pellet is then suspended by vortex mixing in 1 ml of hypertonic medium lacking the macromolecule to be transferred. The resuspended cells are immediately pelleted by centrifugation for 1 min at

high speed in a clinical table-top centrifuge, the hypertonic medium is thoroughly removed using a drawn pasteur pipette, and 0.2–0.7 ml of hypertonic medium containing the macromolecule is added. The cells are suspended by vortex mixing and placed in a 37° water bath for 10 min to allow pinocytic uptake of the macromolecule.

At this point there are two options, depending upon the need to retrieve the molecule being transferred. Either dilute the suspended cells directly in 10 ml of hypertonic medium, allow them to stand for 2 min, and then collect them by centrifugation. Alternatively, centrifuge the cells for 1 min at high speed, collect the hypertonic medium plus macromolecule, and then suspend these cells in 10 ml of hypertonic medium for 2 min. With either procedure, the cells may be collected by centrifugation after 2 min in hypertonic medium, washed in normal culture medium, and cultured for further study.

Osmotic lysis of pinosomes may be repeated several times without severe loss of cell viability. In the original account of the procedure,[2] we subjected L929 cells to four successive rounds of osmotic lysis with 5-min intervals between each round. Almost all cells survived three rounds of osmotic lysis, but 30–40% of the cells died during a fourth round. Presumably, more cells would have survived if longer recovery periods had been interposed between each round of lysis. Thus, it is possible that appropriate spacing of successive rounds of osmotic lysis may permit the transfer of large quantities of macromolecules with little or no cell death.

Measuring the Success of Osmotic Lysis

From the foregoing account it should be clear that the manipulations required for pinosome lysis are extremely simple to perform. It is more difficult, however, to determine whether those manipulations have been successful. If one has access to high-quality fluorescence microscopes, then fluorescent markers of fluid-phase pinocytosis, such as fluoresceinated dextran, may be used. The intracellular distribution of the fluorescent marker can be determined at each stage in the procedure. In my opinion, however, the best assay is provided by horseradish peroxidase (HRP). Unless one is studying a cell line containing high levels of endogenous peroxidase activity, HRP offers both easy cytochemical localization and a reliable quantitative assay for enzyme activity. For this reason, I have included two standard assays based on the work of Steinman *et al.*[3,11]

[11] R. M. Steinman, J. M. Silver, and Z. A. Cohn, *J. Cell Biol.* 63, 949 (1974).

Spectrophotometric Assay for HRP

The reaction mixture is prepared by mixing 18 ml of 50 m*M* sodium phosphate pH 5.0, 2 ml of 0.3% H_2O_2 in 50 m*M* sodium phosphate pH 5.0, and 0.4 ml of *o*-dianisidine at 5 mg per ml in methanol–dimethyl sulfoxide 1 : 1, vol/vol. The assay, typically carried out in 1 ml of reaction mix, is started by addition of enzyme, and the reaction is monitored by an increase in absorbance at 460 nm. The assay is very sensitive; 1 ng of Sigma peroxidase type II will produce a ΔA_{460} of 0.03 per minute at room temperature.

Histochemical Assay for HRP

Cells are fixed in 2% glutaraldehyde in phosphate-buffered saline for 1–10 min, rinsed thoroughly in phosphate-buffered saline, and placed in HRP staining solution for 10–90 min. HRP staining mixture consists of 50 m*M* Tris pH 7.4, containing 0.5 mg per ml diaminobenzidine and 0.01% H_2O_2. HRP is detected by the formation of a golden brown precipitate. HRP-stained cells may be lightly counterstained with Giemsa blood stain to reveal cytological detail.

Expected Results

L929 cells internalize about 10^{-15} liter of hypertonic medium per minute. Since the rate of fluid-phase pinocytosis in these cells is several times greater than that observed for most mammalian cell lines, one should expect uptake rates of about 3×10^{-16} liter per cell per minute. In practical terms this means that, starting with a 100-kDa protein at a concentration of 10 mg per ml in hypertonic medium, approximately 10^5 molecules may be introduced during each round of osmotic lysis. Since fewer than 10^6 copies of most cellular proteins are present per cell, one should generally be able to introduce quantities of protein that are physiologically significant. The vast majority of cells survive a single round of osmotic lysis. We could detect no decrease in plating efficiency of L929 cells, and Ghost *et al.*[9] observed 85–90% survival of CHO cells following lysis of pinocytic vesicles. As mentioned previously, multiple rounds of lysis are possible, but there is a progressive decrease in cell viability. We have also observed that an increased proportion of cells demonstrate nuclear localization of HRP after multiple rounds of osmotic lysis. This may reflect damage to the nuclear membrane.

Manipulating Rates of Pinocytosis

Rates of pinocytosis are under physiological control, and they can be manipulated by growth conditions and various chemicals. Growth factors and secretagogues are of particular interest since they have been shown to stimulate pinocytosis in several cell lines.[12-14] Accordingly, it may be possible to increase pinocytosis with such agents and thereby increase the number of molecules introduced using the osmotic lysis procedure.

Potential Problems

Obviously the molecule to be transferred must be stable in hypertonic medium if the osmotic lysis procedure is to be successful. Whereas most proteins are stable, plasmid DNA is rapidly degraded in culture medium. Serum apparently contains high levels of nucleases, and this may limit the osmotic lysis procedure to proteins and other stable molecules.

A second complication may arise if the molecule to be transferred is readily adsorbed to plasma membranes. Bound molecules may complicate subsequent analysis or binding may prevent transfer of the molecule to the cytoplasm. Thus, it might be necessary to complex unstable or readily adsorbed molecules to other components prior to attempting to transfer them by osmotic lysis.

The pinocytic vesicle and endosome compartments are acidic,[11] and this may also cause problems. For example, we have found that the degradation rate of ^{125}I-bovine serum albumin in HeLa cells is twofold greater after introduction by osmotic lysis as compared to red blood cell-mediated microinjection.[15] Although there are several possible explanations for this enhanced degradation, we suspect that bovine serum albumin is partially denatured in the acidic environment of the pinocytic vesicles.

Despite these potential problems, osmotic lysis of pinosomes offers a very simple procedure for introducing macromolecules into cultured mammalian cells. It should prove useful for studies on a variety of cellular and biochemical phenomena.

[12] H. T. Haigler, J. A. McKanna, and S. Cohen, *J. Cell Biol.* **83**, 82 (1979).
[13] H. S. Wiley and D. D. Cunningham, *J. Cell. Biochem.* **19**, 383 (1982).
[14] H. Koenig, A. D. Goldstone, and C. Y. Lu, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7210 (1983).
[15] B. Tycko and F. R. Maxfield, *Cell* **28**, 543 (1982).
[16] R. A. Schlegel and M. Rechsteiner, *Cell* **5**, 371 (1975).

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Exhibit A

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Reviewer - Nature (Cell Biology)
Reviewer - Journal of Cell Biology
Reviewer - Biology of the Cell
Reviewer - American Journal of Pathology
Reviewer - Blood

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Department of Anatomy, School of Medicine
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"Platelet vimentin - its localization and interactions with the platelet cytoskeleton."

Greater San Francisco Megakaryocyte and Platelet Group, March 7, 1987
"The platelet microtubule coil and microtubule-associated proteins: does the coil maintain platelet shape?"

Greater San Francisco Megakaryocyte/Platelet Society, March 19, 1988
"A morphologic analysis of the cytoskeleton of the amebocytes of the horseshoe crab."

University of California, Davis, Ocular Anatomy - Cell Biology course,
May 24, 1988, "Cellular cytoskeleton."

Greater San Francisco Megakaryocyte/Platelet Society
Lawrence Berkeley Laboratory, March 10, 1990
"Bovine platelets contain an extremely stable microtubule coil cross-linked by a 280 kD microtubule associated protein (MAP)."

Systemix - Palo Alto, CA, March 4, 1992
Bovine megakaryocyte morphogenesis: The role of integrins and the extracellular matrix in proplatelet formation".

Cell and Molecular Biology Division, Lawrence Berkeley Laboratory. Berkeley, CA , June 2, 1992 "Megakaryocyte Morphogenesis *in vitro* is mediated through the vitronectin receptor".

Sacramento Blood Center - Sacramento, CA March 12, 1997
"Membrane phase transition of intact human platelets - correlation with cold-induced

activation".

XVIth Congress of the International Society on Thrombosis and Haemostasis. Florence Italy, June 6-12, 1997.
"Lateral phase separation of membrane phospholipids in platelets during hypothermic storage".

American Society of Hematology December 8, 1997, Transfusion Section
"Anti-freeze glycoproteins inhibit lateral phase separation of platelet membrane lipids during cold storage of washed human platelets".

MedSep Corporation - Covina, CA February 19, 1998
"Role of anti-freeze glycoproteins in platelet stabilization during long-term hypothermic storage".

Novel Approaches to Stabilization of Biomaterials, Breckenridge Colorado, June 9-13, 1999
"Role of membrane microdomains in platelet cold-induced activation"

Comparative Haematopoiesis Gordon Conference, Tilton, N.H. August 8-13, 1999
"Biochemical and Biophysical Analysis of the Megakaryocyte Demarcation Membrane System"

Section of Cardiovascular Medicine, University of California - Davis Medical Center, Sacramento, CA. November 12, 1999. " Cold-induced platelet activation: What do membranes have to do with it?"

National Institutes of Health, Bethesda, MD. June 28, 2000 - "Role of Anti-freeze glycoproteins in the stabilization of platelets for long-term hypothermic storage.

Thermogenesis, Rancho Cordova, CA "State of the Art Freeze-Drying of Human Platelets", July 13, 2001

Biochemistry and Molecular Biology Fall Colloqium, UC-Davis "Cold-Induced Platelet Activation: Phase Transitions and Lipid Rafts", September 27, 2000

Training Integrative Biologists - a Workshop, Merck Research Laboratories, Rahway, New Jersey, October 12, 2000

Pharmacia-Upjohn, Gladstone, NJ "Cold-induced platelet activation, innovative methods for long-term platelet storage. October 19, 2000

Osiris Therapeutics, Baltimore, MD "Freeze-drying human platelets, as a model for long-term storage of mesenchymal stem cells. October 20, 2000

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American Society for Cell Biology, San Francisco, CA " Membrane rafts are a key physiological event in platelet cold-induced activation.". Raft Mini-symposium Session, December 10, 2000

HySeq Inc., Sunnyvale, CA "Membrane rafts are a key physiological even in human platelet activation." March 1, 2001

Society for Cryobiology, Edinburgh Scotland "Successful freeze-drying of platelets with trehalose." July 29, 2001, Symposium - Stabilization of Cells in the Dry State

Cerus Corporation, Concord, CA, "Freeze-dried platelets, trehalose is the key to successful storage.. October 2, 2001

DARPA Annual Conference, Jekyl Island, GA. "Freeze-dried platelets, reach their therapeutic potential. April 1, 2002.

Institute for Laboratory Animal Research, National Academy of Sciences, Washington, D.C. Veterinary Students and Graduate Programs, How do we recruit more students into Ph.D.s? October 4, 2002.

Symposium on Stress Proteins: From Antifreeze to Heat Shock. Bodega Marine Laboratory, University of California – Davis. March 7-9, 2002 "Antifreeze-glycoproteins stabilize platelets and inhibit cold-induced activation."

"Role of Lipid Rafts in Platelet Activation" University of California, Davis, Department of Physiology, School of Medicine April 28, 2003

Promoting the Life Sciences: New Initiatives for the Sacramento Region 2003
By invitation of Governor G. Davis, Sept. 30, 2003

GRADUATE GROUP MEMBERSHIPS:

Cell and Developmental Biology Graduate Group
Comparative Pathology Graduate Group
Biochemistry and Molecular Biology Graduate Group

RESEARCH SUPPORT:

Equine Spleen and Bone Marrow - An Electron Microscopic Analysis. Biomedical Research Support Grant #507. RR05464. Division of Research Resources. NIH - University of Pennsylvania, School of Veterinary Medicine. April 1980 - March 1982 and April 1983 - March 1984.

A Study of Bovine and Equine Platelets: Examination of Microtubule-Associated Proteins, the Microtubule Coil and the Platelet Cytoskeleton.
Biomedical Research Support Grant. UCD #85-28. Division of Research Resources. NIH - University of California, Davis, School of Veterinary Medicine. April 1, 1985 - March 31, 1986.

Isolation and Characterization of Vimentin from Bovine Platelets.
New Faculty Research Award. University of California, Davis. 1985-1986.

Equine Platelets - Cytoskeletal Proteins in Platelet Spreading.
Equine Research Laboratory. School of Veterinary Medicine, University of California, Davis. 1985-1986.

A Study of Bovine Platelets - Interactions Between Microtubule-Associated Proteins and the Microtubule Coil. LDRL Endemic Diseases. School of Veterinary Medicine, University of California, Davis. 1985-1986.

Interactions Between Microtubule-Associated Proteins and Microtubules in Bovine and Equine Platelets. Faculty Research Award. University of California, Davis. 1985-1986.

A Study of Bovine Platelets: Identification and Isolation of Vimentin - Characterization of its Role in Platelet Shape. Biomedical Research Support Grant. UCD #86-23. DivisionResearch Resources. NIH - University of California, Davis, School of Veterinary Medicine. April 1, 1986 - March 31, 1987.

The Role of Vimentin in Platelet Activation. American Heart Association Grant-in-Aid. Dallas, Texas. July 1, 1986 - June 30, 1989. $99,000.

"Platelet Vimentin - Can it Reversibly Associate with the Plasma Membrane." $1,800. 1987, Faculty Research Grant. University of California, Davis.

"Isolation and Culture of Bovine Megakaryocytes: A Model for Platelet Production." $5,000. April 1, 1988 - March 31, 1989. Continuation April 1, 1989 - March 31, 1990, $5,400, Biomedical Research Support Grant. UCD #88-24. Division of Research Resources, National Institutes of Health. University of California, Davis, School of Veterinary Medicine.

"Investigation of Bovine Megakaryocytes: The Role of the Megakaryocyte Cytoskeleton and Cell Shape in Platelet Formation. July 1, 1988 - Sept. 30, 1989, $20,000. Cancer Research Coordinating Committee.

The Role of Microtubules and Microtubule-associated "Motor" Proteins in Normal Bovine Secretion and Endotoxic Shock. July 1, 1989 - June 30, 1990, $3,000. (Continuation 1991-92, $3,000) LDRL.

MAPs 2 and 4 in Platelet Cytoskeletal Reorganization. Bridge Funding, Office of Research, University of California, Davis, May 1991. $15,000.

"Equine platelets: Their Interactions with the Lung in Resting and Strenuously Exercising Horses." 1992-93. $9,249. Equine Research Laboratory.

"The Interaction of Furosemide and Equine Platelets: Morphological and Biochemical Studies." 1992-93. Marcia McDonald Rivas Research Fund.

"Preservation of Platelets by Freeze-Drying." 1994-96. $580,404. Naval Research & Development Command. Funded in collaboration with John Crowe, MCDB, U.C. Davis.

"Vitronectin Receptor Expression in Megakaryocytopoiesis." 1994-96. $300,000. American Cancer Society. Funded in collaboration with Robert Levin, Rush Medical College.

"Platelets and Blood Flow in Sickle Cell Disease" 1995-2000 $589,588. NIH, Funded in collaboration with Ted Wun, UCDMC 5% effort

"Role of Lipid Phase Transition in Platelet Activation" 1997-2003, $1,232,074
NIH, Fern Tablin, P.I.

"Role of AFGPs in Platelet Membrane Stabilization" 1998-2003, $600,000
NIH, Fern Tablin, P.I.

"A Novel Method for Preserving Human Blood Platelets by Freeze-Drying: A Proposal to Develop the Method and Bring it into Clinical Practice" 2000-2003, $467,523, DARPA, Funded in collaboration with John Crowe, P.I., F. Tablin 15% effort

Continuation of above grant 2002-2005: $2,425,596

"Mesenchymal Stem Cell Preservation" 2000-20002 $326,000, DARPA, funded in collaboration with Osiris Therapeutics, and John Crowe, F. Tablin 10% effort

"Development of a Method to Preserve Red Blood Cells by Freeze-Drying,:" 2002-2005 $1,836,800, John H. Crowe and Fern Tablin F. Tablin 10% effort

SELECTED PEER-REVIEWED PUBLICATIONS

Hays, L.M., Feeney, R.E., Tablin, F., Oliver, A.E., Walker, N.E., Crowe, L.M., and J.H. Crowe. Fish anti-freeze glycoproteins protect cellular membranes during lipid-phase transitions. News in Physiological Sciences 12:189-194, 1997.

Wun, T., Paglieroni,T., Tablin,F., Welborn,J., Nelson,K., and A. Cheung. Platelet activation and platelet-erythrocyte aggregates in patients with sickle cell anemia. The Journal of Laboratory and Clinical Medicine 129:507-516, 1997.

Wun, T., Paglieroni,T., Rangaswami,A., Franklin, P.H., Welborn,J., Cheung, A., and F. Tablin. Platelet activation in patients with sickle cell disease. British Journal of Haematology 100:741-749, 1998.

Tablin, F., Rabier, M.J., Walker, N.J., Velasco, V.M., Field, C.L. and R.M. Leven. Tenascin-C is synthesized and secreted by megakaryocytes, whose adherence to intact tenascin is mediated by the integrin subunit B1. Comparative Haematology International 8:142-149, 1998.

Tsvetkova, N.M., Crowe, J.H., Walker, N.J., Crowe, L.M., Oliver, A.E., Wolkers, W.F. and F. Tablin. Physical properties of membrane fractions isolated from human platelets: implications for chilling induced platelet activation. Molecular Membrane Biology 16:265-272, 1999.

Crowe, J.H., Tablin, F., Tsvetkova, N., Oliver, A.E., Walker, N.J. and L.M. Crowe. Are lipid phase transitions responsible for chilling damage in platelets? Cryobiology 38:180-191, 1999.

Wun, T., Paglieroni, T., Field, C.L., Welborn, J., Cheung, A., Walker, N.J., and F. Tablin. Platelet-erythrocyte adhesion in sickle cell disease. J. Investigative Medicine 47:121-127, 1999.

Oliver, A.E., Tablin, F., Walker, N.J. and J.H. Crowe. The internal calcium concentration of human platelets increases during chilling. Biochimica Biophysica Acta 1416:349-360, 1999.

Oliver, A.E., Baker, G.A., Fugate, R.D., Tablin, F. and J.H. Crowe. Effects of temperature on calcium-sensitive fluorescent probes. Biophysical Journal 78:2116-2126, 2000.

Tablin, F., Walker, N.J., Klein, S.D., Field, C.L. and J.H. Crowe. Animal models for studies on cold-induced platelet activation in human beings. Journal of Laboratory and Clinical Medicine 135:339-346, 2000.

Tsvetkova, N.M., Walker, N.J., Crowe, J.H., Field, C.L., Shi, Y. and F. Tablin. Lipid phase separation correlates with activation in platelets during chilling. Molecular Membrane Biology 17:209-218, 2000.

Field, C.L., Walker, N.J., and F. Tablin. Northern elephant seal platelets: analysis of shape change and response to platelet agonists. Thrombosis Research 101:267-277, 2001.

Wolkers, W.F., Walker, N.J., Tablin, F. and J. H. Crowe. Human platelets loaded with trehalose survive freeze-drying. Cryobiology 42:79-87, 2001.

Crowe, J.H., Crowe, L.M., Oliver, A.E., Tsvetkova, N., Wolkers, W. and F. Tablin. The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. Cryobiology 43:89-105, 2001.

Tablin, F., Wolkers, W.F., Walker, N.J., Oliver, A.E., Tsvetkovak, N.M., Gousset, K., Crowe, L.M. and J.H. Crowe. Membrane reorganization during chilling: implications for long-term stabilization of platelets. Cryobiology 43:114-123, 2001.

Tsvetkova, N.M., Phillips, B.L., Krishnan, V.V., Feeney R.E., Fink, W.H.,Crowe, J.H., Risbud, S.H., Tablin, F. and Y.Yeh. Dynamics of antifreeze glycoproteins in the presence of ice. Biophysical Journal 82:464-473, 2002.

Gousset, K., Wolkers, W.F., Tsvetkova, N.M., Oliver, A.E., Field, C.L., Walker, N.J., Crowe, J.H. and F. Tablin. Evidence for a physiological role for membrane rafts in human platelets. Journal of Cellular Physiology 190:117-128, 2002

Tomczak, M.M., Hincha, D.K., Estrada, S.D., Wolkers, W.F., Crowe, L.M., Feeney, R.E., Tablin, F. and J.H. Crowe. A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. Biophysical Journal 82:874-881, 2002.

Wolkers, W.F., Tablin, F. and J.H. Crowe. From anhydrobiosis to freeze-drying of eukaryotic cells. Comparative Biochemistry and Physiology Part A Molecular and Integrative Physiology 131A:535-543, 2002.

Wolkers, W.F., Crowe, L.M., Tsvetekova, N.M., Tablin, F. and J.H. Crowe. In situ assessment of erythrocyte membrane properties during cold storage. Molecular Membrane Biology 19:59-65, 2002.

Cheung, A.T.W., Chen, P.C.Y., Larking E.C., Duong, P.L., Ramanujam, S., Tablin, F. and T. Wun. Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study. Blood 99:2999-4005, 2002

Luria, A., Vegelyte-Avery, V., Smith, B., Tsvetkova, N.M., Wolkers, W.F., Crowe, J.H., Tablin, F. and R. Nuccitelli. Detergent-free domain isolated from Xenopus egg plasma membrane with properties similar to those of detergent resistant membranes. Biochemistry 41:13189-13197, 2002.

Crowe, J.H., Oliver, A.E. and F. Tablin. Is there a single biochemical adaptation to anhydrobiosis? Integrative and Comparative Biology 42:497-503, 2002.

Crowe, J.H., Tablin, F., Wolkers, W.F., Gousset, K., Tsvetkova, N.M., and J. Ricker. Stabilization of membranes in human platelets freeze-dried with trehalose. Chemistry and Physics of Lipids 1122:41-52, 2003.

Tsvetkova, N.M., Horvath, I., Torok, Z., Wolkers, W.F., Balogi,Z., Shigapova,N., Crowe, L.M., Tablin, F., Vierling, E.,

Crowe, J.H. and L. Vigh. Small heat shock proteins regulation membrane lipid polymorphism. Proc. Natl. Acad. Sci. USA 99:13504-13509, 2003.

Wolkers, W.F., S.A. Looper, A.E. McKiernan, N.M. Tsvetkova, F. Tablin and J.H. Crowe. Membrane and protein properties of freeze-dried mouse platelets. Molecular Membrane Biology 19:201-210, 2003.

Ricker, J.V., N.M. Tsvetkova, W.F. Wolkers, C. Leidy, F. Tablin, M. Longo and J.H. Crowe. Trehalose maintains phase separation in an air-dried binary lipid mixture. Biophysical Journal 84:3045-3051, 2003.

Wolkers, W.F., S.A. Looper, R.A. Fontanilla, N.M. Tsvetkova, F. Tablin and J.H. Crowe. Temperature dependence of fluid phase endocytosis coincindes with membrane properties of pig platelets. Biochemica et Biophysica Acta 1612:154-163, 2003.

Wolkers, W.F., N.J. Walker, Y. Tamari, F. Tablin and J.H. Crowe. Towards a clinical application of freeze-dried platelets. Cell Preservation Technology 1:175-188, 2003.

Norris, J.W., M.M. Tomczak, A.E. Oliver, N.M. Tsvetkova, J.H. Crowe, F. Tablin and R. Nuccitelli.Structural characterization of the ADAM 16 disintegrin loop active site. Biochemistry 42:9813-9821, 2003.

Gousset, K., Tsvetkova, N.M., Crowe, J.H. and F. Tablin. Important role of raft aggregation in the signaling events of cold-induced platelet activation. Biochimica et Biophysica Acta 1660:7-15, 2004.

Leidy, C., Gousset, K., Ricker, J., Wolkers, W.F., Tsvetkova,N.M., Tablin, F. and J.H. Crowe. Lipid phase behavior and stabilization of domains in membranes of platelets. Cell Biochemistry and Biophysics 40:123-148, 2004

Oliver, A.E., Jamil, K., Crowe, J.H. and F. Tablin. Loading human mesenchymal stem cells with trehalose by fluid-phase endocytosis. Cell Preservation Technology 2:35-49, 2004

Shrimpton, C.N., Gousset, K., Tablin, F. and J.A. Lopez. Isolation and analysis of platelet lipid rafts. Methods Mol. Biol. 273:213-238, 2004

Ma, X., Jamil, K., Macrae, T.H., Clegg, J.S., Russell, J.M., Villeneuve, T.S., Euloth, M., Sun, Y., Crowe, J.H., Tablin, F. and A.E. Oliver. A small stress protein acts synergistically with trehalose to confer desiccation tolerance on mammalian cells. Cryobiology 51:15-28, 2005

Kheirolomoon, A., Satpathy, G.R., Torok, Z., Banerjee, M., Bali, R., Novaes, R.C., Little, E., Manning D.M., Dwyre, D.M., Tablin, F., Crowe, J.H. and N.M. Tsvetkova. Phospholipid vesicles increase the survival of freeze-dried human red blood cells. Cryobiology 2005 51:290-305.

Tang, M., Wolkers, W.F., Crowe, J.H. and F. Tablin. Freeze-dried rehydrated human blood platelets regulate intracellular pH. Transfusion, 2005 in press.